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The Novel Anticonvulsant Drug, Gabapentin (Neurontin), Binds to the $\alpha_2\delta$ Subunit of a Calcium Channel*

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Gabapentin (1-(aminomethyl)cyclohexane acetic acid; Neurontin) is a novel anticonvulsant drug, with a mechanism of action apparently dissimilar to that of other antiepileptic agents. We report here the isolation and characterization of a [^{3}H]gabapentin-binding protein from pig cerebral cortex membranes. The detergent-solubilized binding protein was purified 1022-fold, in a six-step column-chromatographic procedure, with a yield of 3.9%. The purified protein had an apparent subunit M_r of 130,000, and was heavily glycosylated. The partial N-terminal amino acid sequence of the M_r 130,000 polypeptide, EPFPSAVTIK, was identical to that reported for the $\alpha_2\delta$ subunit of the L-type Ca^{2+} channel from rabbit skeletal muscle (Hamilton, S. L., Hawkes, M. J., Brush, K., Cook, R., Chang, R. J., and Smilowitz, H. M. (1989) *Biochemistry* 28, 7820–7828). High levels of [^{3}H]gabapentin binding sites were found in membranes prepared from rat brain, heart and skeletal muscle. Binding of [^{3}H]gabapentin to COS-7 cells transfected with $\alpha_2\delta$ cDNA was elevated >10-fold over controls, consistent with the expression of $\alpha_2\delta$ protein, as measured by Western blotting. Finally, purified L-type Ca^{2+} channel complexes were fractionated, under dissociating conditions, on an ion-exchange column; [^{3}H]gabapentin binding activity closely followed the elution of the $\alpha_2\delta$ subunit. [^{3}H]Gabapentin is the first pharmacological agent described that interacts with an $\alpha_2\delta$ subunit of a voltage-dependent Ca^{2+} channel.

Gabapentin (1-(aminomethyl)cyclohexane acetic acid; Neurontin) is a novel antiepileptic drug that is orally active in various animal models of epilepsy, including maximal electroshock in rats and pentylenetetrazol- or audiogenically induced seizures in mice (1–3). Gabapentin has also been shown to be effective in decreasing the frequency of seizures in medically refractory patients with partial or generalized epilepsy (3, 4). Although originally synthesized as a lipophilic γ-aminobutyric acid (GABA)[1] analogue, capable of penetrating the blood-brain barrier, gabapentin does not possess a high affinity for either $GABA_A$ or $GABA_B$ receptors, does not influence neural uptake of GABA and does not inhibit the GABA-metabolizing enzyme, GABA transaminase (EC 2.6.1.19) (3, 5). Moreover, gabapentin does not affect voltage-dependent sodium channels (the site of action of several antiepileptic drugs, including phenytoin, carbamazepine, and valproate) and is inactive in assays for a wide range of other neurotransmitter receptors, enzymes, and ion channels (5, 6).

A single high affinity (K_D = 38 ± 2.8 nM) binding site for [^{3}H]gabapentin in rat brain has been described (7). Radioligand binding to brain membranes was potently inhibited by a range of gabapentin analogues and by several 3-alkyl-substituted analogues of GABA, although GABA itself was only weakly active. Other antiepileptic drugs including phenytoin, diazepam, carbamazepine, valproate, and phenobarbitone were inactive. Gabapentin (IC_{50} = 80 nM) and (*RS*)-3-isobutyl-GABA (IC_{50} = 80 nM) were the most active compounds identified (7). The (*S*+)-enantiomer of 3-isobutyl-GABA was significantly more active than the (*R*−)-enantiomer both in displacing [^{3}H]gabapentin binding and in preventing maximal electroshock seizures in mice (8). These data strongly suggest that the protein defined by [^{3}H]gabapentin plays an important role in controlling the excitability of neurons.

Despite extensive research the mechanism of action of gabapentin remains unclear. *In vivo* behavioral studies have suggested the possible involvement of the glycine co-agonist site of the NMDA receptor complex in the anticonvulsant action of gabapentin; intracerebroventricular administration of D-serine (a glycine site agonist) reversed the protection afforded by gabapentin against chemically induced seizures in mice (9). However, radioligand binding assays have not shown gabapentin to inhibit strychnine-insensitive [^{3}H]glycine binding to brain membranes, or to influence the binding of [^{3}H]MK-801 to the NMDA receptor channel (7). Other reports provide some evidence for an interaction between gabapentin and an L-system amino acid transporter; gabapentin crosses the intestinal membrane by a saturable process that is competitively inhibited by leucine (10). Moreover, the binding of [^{3}H]gabapentin to brain membranes is potently inhibited by neutral L-amino acids and moderately inhibited by the L-system substrate, BCH (11). On the other hand, membranes prepared from tissues known to exhibit L-system transport activity (*e.g.* kidney) appear to lack [^{3}H]gabapentin binding sites (7). Thus, the relationship between the L-system transporter and the [^{3}H]gabapentin-binding protein remains unclear.

To identify the molecular target for gabapentin, we have purified and characterized a [^{3}H]gabapentin-binding protein from pig cerebral cortex membranes. Partial N-terminal sequencing identifies the protein as an $\alpha_2\delta$ subunit of a voltage-dependent Ca^{2+} channel. This identification is supported by several lines of evidence, including heterologous expression of

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[1] The abbreviations used are: GABA, γ-aminobutyric acid; BCH, 2-aminobicyclo-[2.2.1]-heptane-2-carboxylic acid; BigCHAP, N,N-bis-(3-D-gluconamidopropyl)cholamide; MK-801, [^{3}H](5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine (Dizolcipine); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

rabbit skeletal muscle $\alpha_2\delta$ cDNA in COS-7 and HEK cells; and radioligand binding and immunoblotting studies, after column fractionation of purified L-type Ca^{2+} channel complexes under dissociating conditions.

EXPERIMENTAL PROCEDURES

Materials—Pig brains were obtained fresh from the local abattoir and transported to the laboratory on ice. All detergents were from Calbiochem Ltd., Nottingham, United Kingdom (UK). Hydroxyapatite was obtained from Jones Chromatography, Hengoed, Mid Glamorgan, UK. All other chromatography media were from Pharmacia Biotech Ltd., Milton Keynes, Bucks, UK. Mini-protean II precast gradient gels, and reagents for silver staining, were from Bio-Rad Laboratories, Hemel Hempstead, Herts, UK. Ultrafiltration cells and YM-10 membranes were from Amicon Ltd., Stonehouse, Gloucester, UK. GF/B filters were from Whatman International, Maidstone, Kent, UK. Glycopeptidase F was from Boehringer Mannheim, Lewes, East Sussex, UK. Lipofectamine and cell culture media were obtained from Life Technologies Ltd., Paisley, Renfrewshire, UK. Monoclonal antibodies raised against rabbit skeletal muscle α_1 (12) and β (13) subunits were from TCS Biologicals Ltd., Botolph Claydon, Bucks, UK. Other standard reagents were obtained from either Sigma, Poole, Dorset, UK or FSA Supplies, Loughborough, Leicestershire, UK. [³H]Gabapentin (57.7 Ci/mmol) was custom synthesized by Amersham International, Amersham, Bucks, UK. [³H]Nitrendipine was obtained from Du Pont Ltd., Stevenage, Herts, UK. Unlabeled gabapentin and the enantiomers of 3-isobutyl GABA were obtained from Parke-Davis, Ann Arbor, MI.

Radioligand Binding Assays—Binding of [³H]gabapentin to soluble preparations was carried out at 22 °C in 10 mM Hepes/KOH, pH 7.4, for 45 min. Assay tubes contained 125 μl of 20 mM Hepes/KOH buffer, 25 μl of protein, 25 μl of [³H]gabapentin (final assay concentration 20 nM), and other additions as required in a final volume of 250 μl. Nonspecific binding was defined as that obtained in the presence of 10 μM (*S*+)-3-isobutyl GABA. Separation of bound and free ligand was effected by rapid filtration through 0.3% polyethyleneimine-impregnated GF/B filters. Filters were washed three times with 4 ml of cold 50 mM Tris/HCl, pH 7.4, and counted in a scintillation counter. Typically, 10 μl of crude soluble brain extract gave total counts of around 4000 dpm, with a background of 250–300 dpm (*i.e.* over 90% specific binding). Binding of [³H]gabapentin to particulate fractions was performed as described by Suman-Chauhan *et al.* (7). [³H]Nitrendipine binding was carried out at room temperature for 30 min in 150 mM NaCl, 2.5 mM $CaCl_2$, 50 mM Tris/HCl, pH 7.4. Reactions were stopped by rapid filtration through GF/B filters as described above. Nonspecific binding was defined as that obtained in the presence of 1 μM nifedipine. Competition binding data were transformed and analyzed using Graphpad INPLOT version 4.03 (Graphpad Software Inc.). Saturation data were analyzed using LIGAND version 3.0 (Elsevier-Biosoft).

Preparation of Detergent-solubilized Pig Brain Membranes—This procedure and all other operations in the purification scheme were carried out at 4 °C unless stated otherwise. Pig brain cortex (up to 35 g) was homogenized in 10 volumes of buffer A (0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM Hepes/KOH, pH 7.4) by six strokes of a glass/Teflon homogenizer at 600 rpm. After removal of the pellet spun at 1000 × *g* for 10 min, the supernatant was centrifuged at 40,000 × *g* for 20 min and the resulting pellet was resuspended in 10 volumes of buffer B (Buffer A without sucrose). Following 30 min of continuous stirring, membranes were pelleted as above and resuspended in approximately three volumes of buffer C (1.25 mM EDTA, 1.25 mM EGTA, 25% glycerol, 12.5 mM Hepes/KOH, pH 7.4) at a concentration of 3 mg of protein/ml. Larger quantities of tissue (>100 g) were processed as indicated above, except that a Waring Blender was used in the initial homogenization step. Solubilization was carried out by combining four volumes of membranes with one volume of 2% Tween 20 and mixing in end-over-end fashion for 1 h. The mixture was centrifuged at 100,000 *g* for 1 h, and the resulting soluble fraction was decanted.

Q-Sepharose Chromatography—Tween 20-solubilized proteins were loaded at 4 ml/min on to a Q-Sepharose FF column (2.6 cm (internal diameter) × 37 cm) equilibrated with 0.08% Tween 20, 10 mM Hepes/KOH, pH 7.4. The column was washed with 250 ml of Hepes buffer before elution with a linear gradient of NaCl (0–750 mM) in a total volume of 1 liter of Hepes buffer. Fractions (10 ml) were collected, and aliquots were assayed for [³H]gabapentin binding activity. Active fractions were operationally defined as those exhibiting binding activity values of >30% peak height.

Lentil Lectin Chromatography—Active fractions from the Q-Sepharose column were pooled, brought to 1 mM $CaCl_2$/1 mM $MnCl_2$ (from a 100× stock solution), and loaded at 10 ml/h on to a lentil lectin-Sepharose 4B column (2.6 cm (internal diameter) × 4 cm) equilibrated in 0.08% Tween 20, 400 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 10 mM Hepes/KOH, pH 7.4. The column was washed with 20 ml of equilibration buffer at 10 ml/h followed by 30 ml of buffer at 20 ml/h. Buffer containing 0.5 M methyl-α-D-mannopyranoside was applied to the column, and the pump was switched off for 2 h. The column was then eluted at a flow rate of 2 ml/h, and 2-ml fractions were collected.

Sephacryl S-400 Chromatography—Active fractions from the lectin column were pooled, concentrated to 8.0 ml by ultrafiltration using a YM-10 membrane, and subjected to gel filtration chromatography. The sample was loaded at 1 ml/min on to a Sephacryl S-400 column (2.6 cm (internal diameter) × 92 cm) equilibrated in 0.08% Tween 20, 150 mM NaCl, 10 mM Hepes/KOH, pH 7.4. Active fractions (10 ml each) were pooled and subjected to two cycles of ultrafiltration and dilution with 1 mM NaCl (final salt concentration ~ 2 mM).

Hydroxyapatite Chromatography—The protein was loaded at 7 ml/h on to a hydroxyapatite column (1 cm (internal diameter) × 5.7 cm) equilibrated in 0.08% Tween 20, 1 mM Na_2HPO_4/H_3PO_4, pH 7.4. After washing with 20 ml of 1 mM NaCl containing 0.08% Tween 20, the column was eluted at 5 ml/h with a linear gradient of 0–0.2 M Na_2HPO_4/H_3PO_4, pH 7.4, in a total volume of 100 ml of equilibration buffer. The high salt solution was maintained at 60 °C in a water bath to prevent crystallization. Active fractions (2 ml each) were pooled and brought to 150 mM NaCl from a 5 M stock solution.

Wheat Germ Lectin Chromatography—The sample from the hydroxyapatite column was loaded at 4 ml/h onto a wheat germ lectin-agarose column (1 cm (internal diameter) × 5 cm) equilibrated with 0.08% Tween 20, 450 mM NaCl, 10 mM Hepes/KOH, pH 7.4. The column was washed with 6 ml of equilibration buffer and eluted with 0.35 M *N*-acetyl-D-glucosamine in equilibration buffer using the two-step procedure outlined above for the lentil lectin column. Active fractions (2 ml each) were pooled and diluted (1:5) with 0.08% Tween 20, 10 mM Hepes, pH 7.4 (at 22 °C), before application to a high resolution ion exchange column.

Mono Q Chromatography—After filtration through a 0.2 μM filter, the sample was loaded by means of a 50-ml Superloop on to Mono Q HR 5/5 column equilibrated with 0.08% Tween 20, 10 mM Hepes/KOH, pH 7.4, at 22 °C. Bound proteins were eluted with a linear NaCl gradient (0–750 mM) in a total volume of 50 ml of buffer A. Fractions (1 ml each) were collected and aliquots analyzed in the radioligand binding assay and by SDS-polyacrylamide gel electrophoresis.

Purification of Skeletal Muscle Ca^{2+} Channels—Membranes were prepared from fresh muscle tissue following the method of Nakayama *et al.* (14). Detergent solubilization was performed as described for brain membranes (see above) except that digitonin (1% final concentration) was used. Ca^{2+} channel complexes were purified by sequential chromatography on wheat germ lectin-agarose and Mono Q. Column buffers were identical to those described above, except that digitonin (0.1%) replaced Tween 20.

Deglycosylation with Glycopeptidase F—Aliquots (50 μl) of purified [³H]gabapentin-binding protein were mixed with 25 μl of either 0.5% SDS, 0.1 M 2-mercaptoethanol or water and heated for 5 min at 100 °C. After this, the following additions were made: 25 μl of 0.5 M Tris/HCl, pH 8.0, 10 μl of 0.1 M EDTA/NaOH, pH 8.0, 10 μl of 10% Tween 20, pH 8.0, and either 0.5 μl or 2.5 μl of glycopeptidase F (200 units/ml) or water for controls. Deglycosylation was carried out for 18 h at 37 °C, after which samples were analyzed by SDS-polyacrylamide gel electrophoresis.

Protein Sequencing—The purification procedure was modified to allow the production of sufficient material for N-terminal protein sequencing. Briefly, the hydroxyapatite and wheat germ lectin columns were replaced with a single Cu^{2+}-charged iminodiacetic acid-Sepharose column. Full details of this modified procedure will appear elsewhere.[2] The final sample of purified [³H]gabapentin-binding protein (5 μg) was electrophoresed on a 10% SDS-polyacrylamide gel and electroblotted on to an Immobilon P membrane. The blot was stained with Coomassie Blue and the M_r 130,000 band excised and sequenced on an Applied Biosystems 477A sequencer.

Stable Expression of cDNA in HEK 293 Cells—HEK 293 cells were transfected using Lipofectamine-mediated transfection with two pcDNA3 derivatives, one containing rabbit skeletal muscle $\alpha_2\delta$ cDNA and the other containing human neuronal β subunit cDNA. Stable cell lines were selected using G418 at a concentration of 600 μg/ml medium.

[2] J. P. Brown, V. U. K. Dissanayake, A. Briggs, and N. S. Gee, manuscript in preparation.

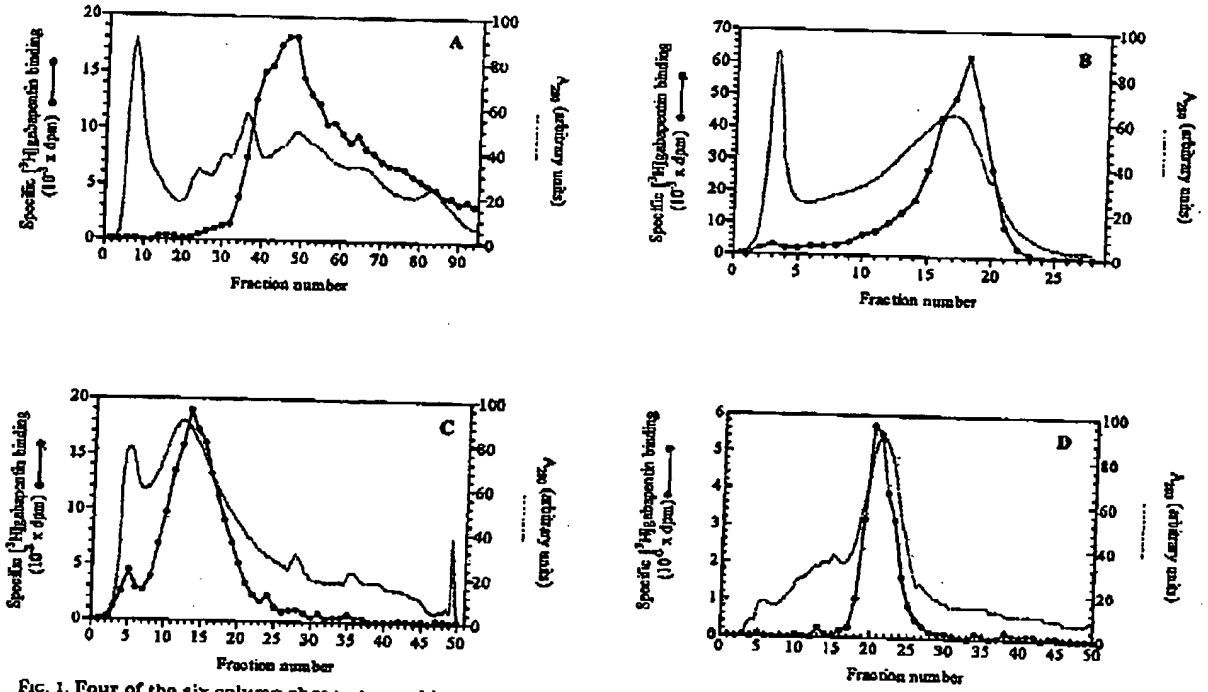


FIG. 1. Four of the six column-chromatographic steps of the purification scheme. *A*, Q-Sepharose; *B*, Sephacryl S-400; *C*, hydroxyapatite; *D*, Mono Q HR 5/5. ●, binding activity; - - -, A_{280}.

These cells were originally developed for studies on recombinant Ca^{2+} channels with different α_1 subunits, hence the co-expression of $\alpha_2\delta$ and β subunits. The cell line designated 2L contained high levels of $\alpha_2\delta$ and β mRNA and protein as measured by Northern and Western blotting, respectively. Membranes were prepared from 2L and HEK 293 cells by hypotonic lysis and homogenization. Extracts were centrifuged at 3000 × *g*, and the resulting supernatant was centrifuged at 50,000 × *g* to pellet membranes. Cell membranes were assayed for [^{3}H]gabapentin binding activity as described above.

Transient Expression of $\alpha_2\delta$ cDNA in COS-7 Cells—COS-7 cells in Dulbecco's modified Eagle's medium, 10% fetal bovine serum were seeded at 3.8×10^5 cells/60-mm Petri dish. Dishes were incubated for 18 h at 37 °C in an atmosphere of 5% CO_2 in air and transfected with 5 μg of plasmid DNA by Lipofectamine-mediated transfection. After another 48 h, cell membranes were prepared and assayed for [^{3}H]gabapentin binding activity as described above.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed in 4–20% gradient gels using the Laemmli (15) buffer system. Gels were stained using the Bio-Rad silver-staining kit according to the manufacturer's instructions.

Western Blotting—Proteins were electrophoretically transferred to nitrocellulose sheets at 25 mA for 72 h according to the method of Towbin *et al.* (16). Blots were incubated with blocking buffer (2% milk powder in 50 mM Tris, 150 mM NaCl, pH 7.5), for 30 min, before incubation with primary antibody for 18 h at 4 °C. Blots were washed five times with blocking buffer, over 15 min, before addition of horseradish peroxidase-conjugated second antibody. After 3 h, blots were washed as above, followed by three rinses in buffer lacking milk powder. Peroxidase activity was detected either using 3-amino-9-ethylcarbazole substrate, as described by Graham *et al.* (17), or by chemiluminescence using a kit supplied by Amersham International.

Protein Determinations—Protein concentration was determined by the method of Bradford (18) using bovine serum albumin as a standard.

RESULTS

Solubilization of the [^{3}H]Gabapentin-binding Protein—Although most studies on the [^{3}H]gabapentin-binding protein have been carried out in the rodent (7, 8, 19), a species such as the pig has obvious advantages as a tissue source for protein purification work. Binding of [^{3}H]gabapentin to membranes prepared from various pig brain regions was therefore examined. Specific activity values for occipital cortex, parietal cortex, frontal cortex, hippocampus, striatum, thalamus, cerebellum, and brain stem were 835, 691, 509, 399, 323, 278, 179, and 123 dpm/mg, respectively. Membranes prepared from whole cerebral cortex were used in all subsequent experiments. Treatment of membranes with solutions of either high salt (up to 4 M NaCl) or EDTA (up to 50 mM) released <3% of the [^{3}H]gabapentin binding sites. These data suggest that the [^{3}H]gabapentin-binding protein is probably integral to the membrane. Several detergents proved to be effective solubilizing agents: 0.1% Tween 20, 0.2% Triton X-114, 0.2% Triton X-100, 0.5% BigCHAP, and 0.4% Brij 35 solubilized 52.3 ± 5.0% (n = 3), 49.2 ± 16.3% (n = 3), 34.3% (n = 2), 41.0% (n = 2), and 33.7 ± 5.7% (n = 3) of the [^{3}H]gabapentin binding sites, respectively. Dissociation constants (K_D values) for the soluble fractions were in the range 32.7–68.9 nM, comparable to the values obtained with brain membranes (values in the range 35–53 nM for six different batches). Tween 20 was used for large scale purifications, as it gave a soluble fraction of a higher specific activity than any of the other detergents tested (data not shown).

Purification of the [^{3}H]Gabapentin-binding Protein—In the purification procedure the chromatographic columns were ordered to maximize resolution and minimize the number of intermediate conditioning steps. Solubilization of brain membranes in a low ionic strength buffer with Tween 20 (a non-ionic detergent) allowed direct application of the crude 100,000 × *g* supernatant on to an anion-exchange column. Two batches of extract, each prepared from 250 g of pig cerebral cortex, were processed on Q-Sepharose FF. Fig. 1*A* shows a typical gradient

TABLE I

Purification of the [³H]gabapentin-binding protein

Purification was as described under "Experimental Procedures." Binding activity was determined using 20 nM [³H]gabapentin. ND, not determined; aliquot of pooled material that was saved for binding assays was inactivated by freezing (see "Results").

Fraction	Total activity	Total protein	Specific activity	Yield	Purification
	pmol	*mg*	*pmol/mg*	%	*-fold*
Membranes	3504	2258	1.55	100	1
Solubilized membranes	2476	449	5.51	70.7	3.55
Q-Sepharose	1115	92.1	12.1	31.8	7.81
Lentil lectin	599	10.4	57.6	17.1	37.2
Sephacryl S-400	376	2.32	162	10.7	105
Hydroxyapatite	ND	0.420	ND	ND	ND
Wheat germ lectin	191	0.223	857	5.45	553
Mono-Q (Fractions 18–25)	136	0.118	1153	3.88	744
Mono-Q (Fractions 20/21)	49.1	0.031	1584	1.40	1022

elution profile obtained with this column. The peak of [³H]gabapentin binding activity emerged at around 400 mM NaCl, although considerable trailing was usually observed. Re-chromatography of the later eluting fractions also yielded a peak of activity at 400 mM NaCl, suggesting that the trailing phenomenon was not due to protein heterogeneity. Further chromatography was performed on lentil lectin-Sepharose, after addition of Ca^{2+} and Mn^{2+} ions (each 1 mM) to the pooled Q-Sepharose extract. These ions caused little or no interference in the [³H]gabapentin binding assay. Equilibration of the lentil lectin column with sugar-containing buffer for 2 h before elution resulted in a sharper peak of binding activity, and thus facilitated chromatography by gel filtration (Fig. 1*B*). The active material from the Sephacryl S-400 column was subjected to two cycles of ultrafiltration and dilution, to reduce the ionic strength of the sample, before application to the hydroxyapatite column. This method of salt removal was much faster than dialysis and gave a higher recovery of [³H]gabapentin-binding protein. While fractionation on hydroxyapatite (Fig. 1*C*) was straightforward, provided the elution buffer was prevented from crystallizing, the eluted material could not be frozen without a substantial loss of [³H]gabapentin binding activity. This phenomenon was not investigated systematically, but the degree of inactivation appeared to be related to the rate of sample cooling. As a precaution, we avoided freezing samples in phosphate buffer and applied the pooled active material directly on to the wheat germ lectin-agarose column. Further chromatography on Mono Q (Fig. 1*D*) allowed the removal of the sugar-containing buffer from the previous step and most of the remaining protein impurities. The progress of one purification is summarized in Table I. The specific activity value for the peak Mono Q fractions was 1584 pmol/mg, which corresponds to a 1022-fold purification over the starting membranes. A lower value of 1153 pmol/mg was calculated for the relatively dilute total Mono Q pool. Stepwise recoveries were in the range 45–72%, and the overall yield was 3.9% or 0.24 μg of gabapentin-binding protein/g (wet weight) of pig cerebral cortex.

Biochemical and Molecular Properties of the Purified [³H]Gabapentin-binding Protein—As shown in Fig. 2, the predominant species in the Mono Q eluent was a polypeptide with an apparent subunit M_r of 130,000. An additional, faint, diffuse band (M_r 25,000), whose intensity paralleled that of the M_r 130,000 band could be visualized if gels were either overloaded or stained twice (data not shown). Under non-reducing conditions, neither of these bands was observed; instead, the purified protein migrated as a single species with an apparent M_r of 170,000 (data not shown). The [³H]gabapentin-binding protein is therefore composed of non-identical subunits covalently linked by at least one disulfide bridge. Gel filtration of the purified protein on a Superose 6 column yielded a molecular size for the Tween 20-[³H]gabapentin-binding protein complex of 260 kDa (n = 3). This is broadly consistent with a species of

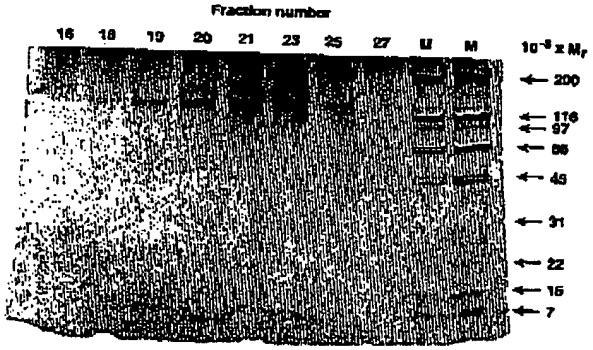


FIG. 2. SDS-polyacrylamide gel electrophoresis of purified [³H]gabapentin-binding protein eluted from Mono Q. *M*, marker proteins. Apparent subunit M_r values (× 10^{-3}) of the markers are indicated.

170-kDa binding to a micello of detergent. However, chromatography of Tween 20-solubilized membrane proteins yielded a molecular size for the [³H]gabapentin-binding protein of 430 kDa (n = 4). Preliminary experiments suggest that the alteration in molecular size occurs during Q-Sepharose chromatography (data not shown).

The retention of the [³H]gabapentin-binding protein by lectin columns suggested that the protein was probably glycosylated. Incubation of the reduced SDS-denatured protein with high concentrations of glycopeptidase F led to a decrease in the apparent subunit M_r from 130,000 to 105,000 (Fig. 3). Purified [³H]gabapentin-binding protein that had been subjected to heat denaturation without SDS and 2-mercaptoethanol was resistant to the enzyme. Thus the [³H]gabapentin protein is heavily glycosylated, with *N*-linked oligosaccharide chains that are accessible to glycopeptidase F only under fully denaturing conditions.

Pharmacological Properties of the Purified Protein—Several compounds were evaluated in competition assays with [³H]gabapentin using the purified [³H]gabapentin-binding protein (Fig. 4). (*S*+)-3-Isobutyl GABA potently inhibited [³H]gabapentin binding with an IC_{50} value of 40 nM (*cf.* gabapentin, IC_{50} = 50 nM), while the (*R*−)-enantiomer was nearly an order of magnitude less effective (IC_{50} = 370 nM). A stereoselective requirement at the [³H]gabapentin binding site was even more pronounced for the L- and D-enantiomers of leucine (IC_{50} values of 80 and 10,000 nM, respectively). The paradigm *L*-system substrate, BCH, displaced [³H]gabapentin binding with an IC_{50} of 691 nM. Similar results to these were obtained both with brain membranes and with Tween 20-solubilized preparations. Thus, the pharmacological characteristics of the

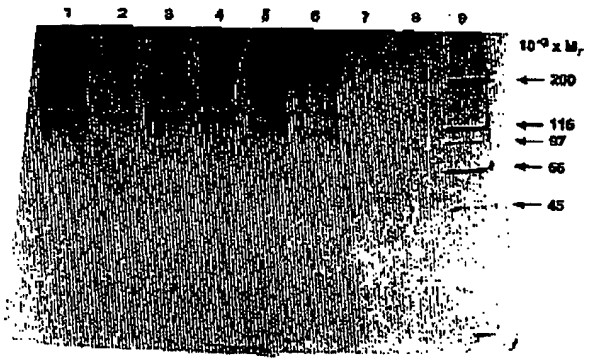


FIG. 3. SDS-polyacrylamide gel electrophoresis of [³H]gabapentin-binding protein showing the effect of glycopeptidase F treatment. Prior to deglycosylation, samples of [³H]gabapentin-binding protein (*lanes 1–6*) or buffer (*lanes 7* and *8*) were boiled in either the absence (*lanes 1–3* and *8*) or the presence (*lanes 4–7*) of SDS/2-mercaptoethanol. *Lanes 1* and *4*, no enzyme; *lanes 2* and *5*, 0.1 unit of enzyme; *lanes 3* and *6–8*, 0.5 unit of enzyme.

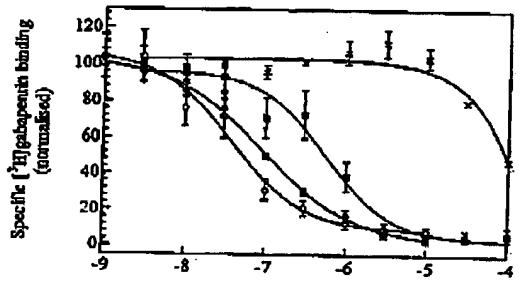
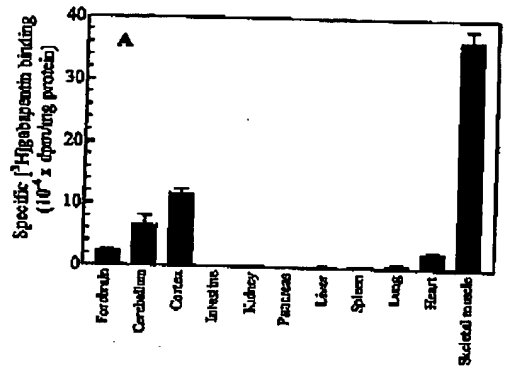


FIG. 4. Inhibition of [³H]gabapentin binding to the purified protein. Various amino acids and related compounds were tested for their ability to inhibit radioligand binding. Each point in the dose-response curves represents the mean from three separate experiments. ○, (*S*+)-3-isobutyl GABA (IC_{50} = 40 nM); ■, (*R*−)-3-isobutyl GABA (IC_{50} = 370 nM); ●, L-leucine (IC_{50} = 80 nM); ×, D-leucine (IC_{50} = 10,000 nM).

[³H]gabapentin-binding protein appear to have been preserved following detergent solubilization and subsequent isolation of the protein.

N-terminal Sequencing and Identification of the [³H]Gabapentin-binding Protein—The availability of microgram quantities of highly purified pig brain [³H]gabapentin-binding protein allowed us to obtain a partial N-terminal sequence for the M_r 130,000 polypeptide. Two preparations of protein were analyzed, and, in each case, 10 cycles of readable sequence were obtained. Both samples gave the same sequence: EPFPSAVTIK. A homology search showed that the N-terminal sequence was identical to that reported for the $\alpha_2\delta$ subunit of the rabbit skeletal muscle voltage-dependent L-type Ca^{2+} channel (20). The same stretch of amino acids is also found in the sequence deduced by cDNA cloning for the human brain $\alpha_2\delta$ Ca^{2+} channel subunit (21). The corresponding sequence for the rat brain $\alpha_2\delta$ sequence contains a prolyl instead of an alanyl residue at position 6 (22).

Tissue Distribution of [³H]Gabapentin-binding Sites in the Rat—The binding of [³H]gabapentin to membranes prepared from some 14 rat tissues was examined. As shown in Fig. 5*A*, the highest level of [³H]gabapentin binding sites was observed in skeletal muscle. Significant levels were found in cerebral cortex, cerebellum, forebrain, and heart. Trace amounts of



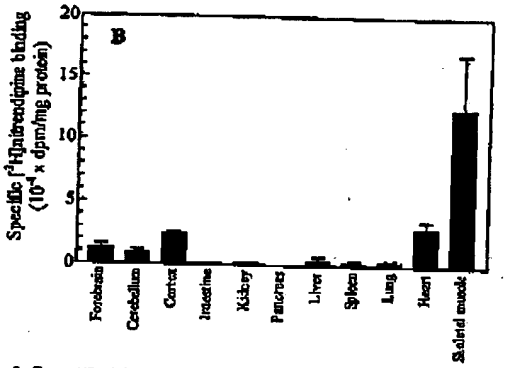


FIG. 5. Specific binding of [³H]gabapentin (*A*) and [³H]nitrendipine (*B*) to membranes prepared from various rat tissues. The results represent mean values ± S.E. of three separate experiments performed in duplicate.

[³H]gabapentin binding sites were found in lung, spleen, liver, and kidney, but pancreas and intestine were devoid of activity. In rat muscle membranes [³H]gabapentin bound to a single population of sites with high affinity (K_D = 29 nM), similar to that reported for rat brain (8). Competition experiments with (*S*+)- and (*R*−)-3-isobutyl-GABA, D- and L-leucine, BCH, and gabapentin gave a rank order of potency that was identical to that obtained with the purified pig [³H]gabapentin-binding protein (see Fig. 4). The distribution of dihydropyridine-sensitive L-type Ca^{2+} channels as defined by [³H]nitrendipine (Fig. 5*B*) was similar, although not identical, to that for $\alpha_2\delta$ subunits labeled by [³H]gabapentin.

Heterologous Expression of $\alpha_2\delta$ cDNA—Binding of [³H]gabapentin to 2L cells, which express the rabbit skeletal muscle $\alpha_2\delta$ protein and a human neuronal β subunit, and to parental HEK 293 cells, was examined. As shown in Fig. 6*A*, specific [³H]gabapentin binding to 2L cell membranes was approximately 85-fold higher than to HEK 293 cell membranes. The trace levels of specific [³H]gabapentin binding to HEK 293 cells (and to kidney membranes; Fig. 5*A*) is probably explained by low level expression of endogenous Ca^{2+} channel subunits. Indeed, splice variants of $\alpha_2\delta$ have been detected in HEK 293 cells by polymerase chain reaction methods (23). COS cells transfected with pcDNA3/$\alpha_2\delta$ cDNA expressed >10-fold higher levels of [³H]gabapentin binding sites than COS cells transfected with vector alone (Fig. 6*A*). This was consistent with increased expression of $\alpha_2\delta$ protein as measured by Western blotting (Fig. 6*B*). The electrophoretic properties of the ex-

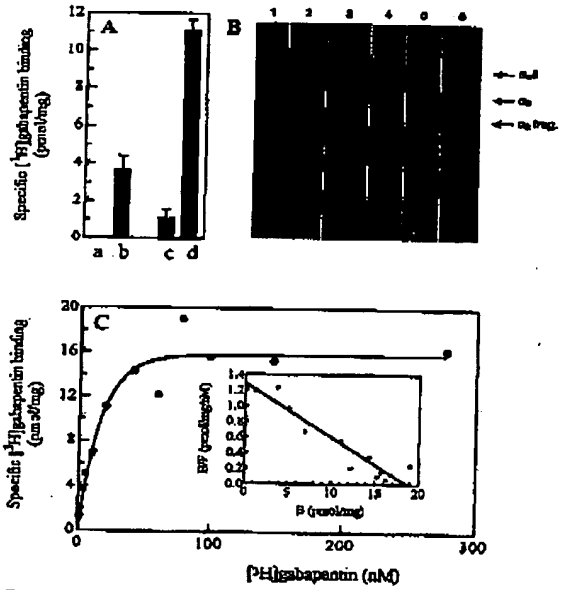


FIG. 6. *A*, binding of [³H]gabapentin to HEK (*a*), 2L (*b*), and COS-7 (*c* and *d*) cell membranes. The results represent the mean values ± S.E. of four separate experiments performed in duplicate (HEK and 2L cells) or two experiments with replicates of six (COS-7 cells). The 2L line stably expresses $\alpha_2\delta$ and β subunits and is a derivative of HEK 293. COS cells were transfected either with pcDNA3 alone (*c*) or with a pcDNA-3/$\alpha_2\delta$ construct (*d*). The concentration of [³H]gabapentin in the COS-7 experiments was 60 nM. *B*, immunoblotting of COS-7 cell membranes using an affinity-purified anti-α_2 antibody. Membranes were electrophoresed either under reducing (*lanes 1–3*) or non-reducing (*lanes 4–6*) conditions. *Lanes 1* and *4*, sample c (5 μg of protein); *lanes 2* and *5*, sample d (5 μg of protein); *lanes 3* and *6*, 200 ng of purified pig brain [³H]gabapentin-binding protein. α_2 *frag.* marks the position of a proteolytic degradation product of the pure protein. *C*, Scatchard analysis of binding data obtained with COS-7 cell membranes (sample d). The data shown are from one representative experiment.

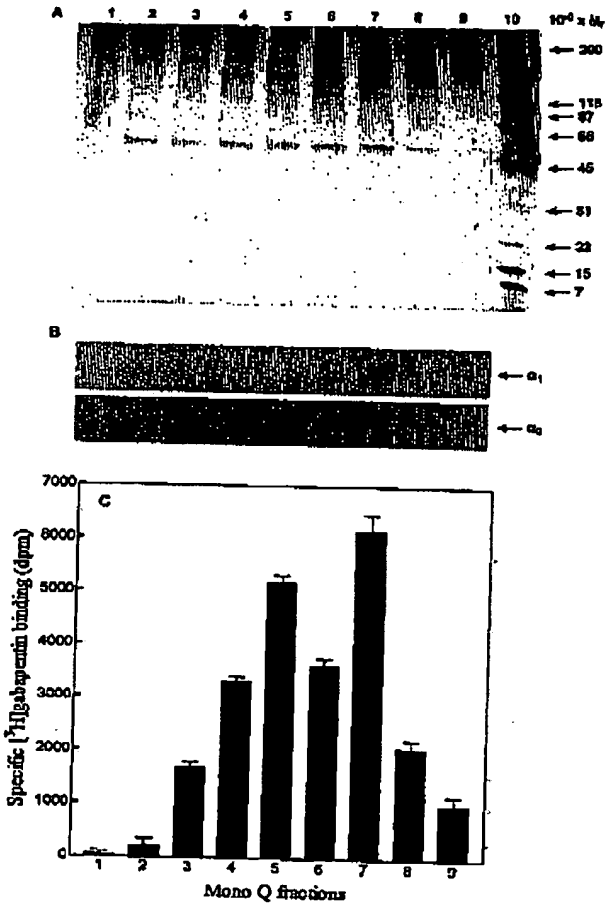


FIG. 7. *A*, silver-stained SDS-polyacrylamide gel of skeletal muscle L-type Ca^{2+} channels purified on Mono Q. The column was eluted with a linear NaCl gradient (0–750 mM), and 1-ml fractions were collected (total gradient volume of 75 ml). *Lanes 1–9* correspond to fraction numbers 24, 28, 32, 36, 40, 44, 48, 52, and 56, respectively; a 20-μl aliquot of each fraction was analyzed; *lane 10*, molecular weight markers. *B*, immunoblotting of Mono Q column fractions. Nitrocellulose blots were prepared from two gels, each identical to the one described above. Blots were probed either with a monoclonal antibody against the skeletal muscle α_1 subunit or with a polyclonal antibody raised against the pig brain α_2 subunit. Only the relevant portion of each blot is shown. *C*, binding of [³H]gabapentin to Mono Q fractions. Assays were performed in triplicate (20 μl of sample/assay) with 20 nM [³H]gabapentin.

pressed $\alpha_2\delta$ protein were consistent with those reported for the skeletal muscle protein (24). In COS-7 cells transfected with $\alpha_2\delta$ cDNA, [³H]gabapentin bound to a single population of sites, with high affinity (K_D = 13 nM), with a maximum binding capacity of 17.8 pmol/mg of protein (Fig. 5*C*). Similar results to those were obtained in a second transfection experiment (K_D = 19.8 nM; B_{max} = 25.9 pmol/mg).

Fractionation of Partially Dissociated Ca^{2+} Channel Complexes—Ca^{2+} channel complexes were purified from digitonin-solubilized skeletal muscle membranes by sequential chromatography on wheat germ lectin-agarose and Mono Q. Fig. 7*A* shows a silver-stained SDS-polyacrylamide gel of the Mono Q eluent (lanes correspond to every fourth fraction). An unequivocal assignment of the α_1 and α_2 polypeptides, which are visible on the SDS gel, was achieved by immunoblotting (Fig. 7*B*). The α_1 and $\alpha_2\delta$ subunits showed markedly different elution profiles, consistent with partial disruption of the Ca^{2+} channel complexes. [³H]Gabapentin binding activity, which eluted as two overlapping peaks (Fig. 7*C*), closely followed the elution of the $\alpha_2\delta$ polypeptide. The α_1 subunit eluted as a single peak, coincident with the second peak of [³H]gabapentin binding activity. The two $\alpha_2\delta$-containing peak fractions, and the material applied to the Mono Q column, were further examined by immunoblotting using a higher gel loadings. In the case of the β subunit, which could not be visualized using 3-amino-9-ethylcarbazole substrate, a more sensitive luminol-based detection system was used. Fig. 8 shows that the material applied to the Mono Q column contained α_1, $\alpha_2\delta$, and β subunits. Like the α_1 subunit, the β subunit was found only in the second peak of [³H]gabapentin binding activity. The amount of [³H]gabapentin binding activity in the three samples was broadly consistent with the amount of $\alpha_2\delta$ polypeptide as measured by immunoblotting.

DISCUSSION

The purification of the detergent-solubilized [³H]gabapentin-binding protein from pig brain was achieved by sequential chromatography on Q-Sepharose, lentil lectin, Sephacryl S-400, hydroxyapatite, wheat germ lectin, and Mono Q. The protein in the Mono Q eluent was analyzed on a 4–20% gradi-

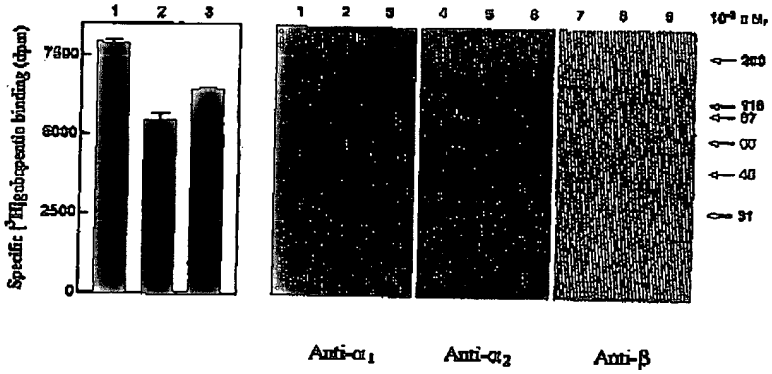


FIG. 8. Binding assays and immunoblotting of pooled wheat germ eluent Mono Q peak fractions. Nitrocellulose blots were prepared from three identical SDS-polyacrylamide gels. *Lane 1*, wheat germ eluent (5 μl); *lane 2*, Mono Q peak 1 (40 μl); *lane 3*, Mono Q peak 2 (40 μl). Each blot was probed with a different primary antibody and an appropriate horseradish peroxidase-conjugated second antibody. Peroxidase activity was detected using 3-amino-9-ethylcarbazole (α_1 and $\alpha_2\delta$ subunits) or luminol (β subunit). [³H]Gabapentin binding assays on the same samples were performed in triplicate.

ent SDS-polyacrylamide gel, which covers a broad range of molecular weights. The amount of radioligand binding paralleled the intensity of the M_r 130,000 band, although other faint co-eluting bands were observed. The possibility that [³H]gabapentin might bind to one of these quantitatively minor components was excluded in other experiments (see below). The final specific activity, based on the peak Mono Q fractions, was 1584 pmol/mg. This value is approximately 5-fold less than that expected, given a starting specific activity value of 1.55 pmol/mg for brain membranes. However, the low concentration of protein in the Mono Q eluent, and the presence of Tween 20, which interfered with the protein assay, precluded an accurate determination of specific activity. The susceptibility of the protein to inactivation, which was particularly evident in phosphate buffers, may also have been a contributory factor. In all, we have prepared five batches of [³H]gabapentin-binding protein using this, or a very similar, procedure. Preparations of higher purity, though with reduced microgram yields, could be obtained either by reducing the amount of starting material, or by judicious selection of fractions to be pooled. However, the purification scheme described here offers a reasonable compromise between yield and purity.

The partial N-terminal sequencing of the M_r 130,000 polypeptide was a crucial step in the identification of the [³H]gabapentin-binding protein as an $\alpha_2\delta$ subunit of a Ca^{2+} channel. The sequence homology with the $\alpha_2\delta$ subunit predicted a more widespread tissue distribution for the [³H]gabapentin-binding protein than has previously been acknowledged (7). Indeed, radioligand binding assays revealed high concentrations of [³H]gabapentin binding sites not only in brain, but also in skeletal muscle and heart. The distribution of [³H]nitrendipine sites was broadly similar to that for [³H]gabapentin, although detailed differences in the relative levels of the binding sites were apparent. However as [³H]gabapentin probably labels both L- and non-L-type voltage-dependent Ca^{2+} channels, these differences are not unexpected. To further confirm the identity of the [³H]gabapentin-binding protein, we performed radioligand binding assays on cell lines expressing the $\alpha_2\delta$ subunit from rabbit skeletal muscle. High levels of specific [³H]gabapentin binding in the 2L cell line, which expresses both $\alpha_2\delta$ and β calcium channel subunits, were observed, whereas the parental HEK 293 line was almost devoid of activity. COS-7 cells transfected with $\alpha_2\delta$ cDNA alone, expressed >10-fold higher levels of [³H]gabapentin binding sites than control cells. These data were consistent with expression of the $\alpha_2\delta$ protein, as measured by Western blotting using a polyclonal antibody raised against the α_2 polypeptide. The expressed $\alpha_2\delta$ protein bound [³H]gabapentin with high affinity (K_D = 16 nM; n = 2), broadly similar to that determined for rat muscle membranes. These studies argue strongly that [³H]gabapentin binds to the $\alpha_2\delta$ subunit in our purified preparation and not to a minor contaminant.

Most studies on the structure and functional domains of Ca^{2+} channels have focused on the L-type channel from rabbit skeletal muscle. This channel is a heteromultimeric complex composed of an α_1 subunit, which forms the Ca^{2+} conducting pore, and three accessory subunits: $\alpha_2\delta$, β, and γ. Although it is now well established that the α_1 subunit is the target for dihydropyridines, early preparations of the "dihydropyridine receptor" apparently contained only an $\alpha_2\delta$ subunit (25). It is now known that these samples were "contaminated" with α_1 subunits that were not visible on SDS gels. In view of the heterologous expression studies described above, the possibility of [³H]gabapentin's binding to α_1 subunits in our preparation of purified pig brain $\alpha_2\delta$ was unlikely. However, $\alpha_2\delta$ and β subunit cDNAs have been shown to enhance the cell surface expression of *co-transfected* α_1 subunits (21, 23, 26). We considered the possibility that transfection of $\alpha_2\delta$ cDNA might enhance the expression of *host-derived* α_1 subunits. To address this, we isolated L-type Ca^{2+} channel complexes from rabbit skeletal muscle membranes. Digitonin was employed as the solubilizing agent, as it is reported to preserve the oligomeric structure of Ca^{2+} channels (20). Purification was achieved using a combination of lectin chromatography and ion-exchange chromatography, as used by others (20, 25, 27). To allow fractionation of individual subunits, we planned to disrupt the Ca^{2+} channel complexes by exchanging detergents. However, we found that this step was not necessary; partial dissociation occurred during chromatography on Mono Q, even in digitonin-containing buffers. Two peaks of [³H]gabapentin binding activity, which closely followed the elution of the $\alpha_2\delta$ polypeptide, were observed. The α_1 and β subunits were found only in the second peak, presumably as $\alpha_1\beta$ and $\alpha_1\beta\alpha_2\delta$ complexes. The earlier elution, from ion-exchange columns, of dissociated $\alpha_2\delta$ subunits is in agreement with another study (20). The profile of the γ subunit was not assessed by immunoblotting, although a faint band of the expected size (35 kDa) was seen in the peak α_1 fraction (Fig. 7*A*, *lane 7*). Data from heterologous expression studies and purification experiments show conclusively that the single high affinity [³H]gabapentin binding site found in brain and muscle membranes is the $\alpha_2\delta$ subunit. Moreover, it is clear that the binding of [³H]gabapentin does not require the presence of the α_1 and β subunits. However, these subunits may modulate the binding of [³H]gabapentin to the $\alpha_2\delta$ subunit in hetero-oligomeric complexes. A slight enhancement of binding to the $\alpha_2\delta$ subunit, in the presence of the α_1 and β subunits, is suggested from the data in Fig. 7 (compare *lanes 5* and *7*) and Fig. 8 (α_2 immunoblot; compare *lane 2* with *lanes 1* and *3*). It is

interesting to note that the $\alpha_2\delta$ subunit itself modulates the binding of ω-conotoxin to the α_1 subunit of the N-type Ca^{2+} channel (23).

The biochemical properties of the purified pig brain [³H]gabapentin-binding protein are strikingly similar to those of the $\alpha_2\delta$ subunit from rabbit skeletal muscle: the muscle $\alpha_2\delta$ subunit exhibits a characteristic mobility shift on SDS-polyacrylamide gels, in the presence and absence of reducing agents (24, 27); moreover, the removal of *N*-linked carbohydrate from the α_2 subunit, by exhaustive digestion with glycopeptidase F, results in an apparent molecular mass of 105 kDa (28). The $\alpha_2\delta$ subunit is thought to be anchored to the membrane by a hydrophobic segment located in the 25-kDa δ peptide (24), although transmembrane segments in the larger 140-kDa α_2 component have also been postulated (29). Although we have yet to confirm the identity of the 25-kDa component in our purified preparation by N-terminal sequencing, the poor staining of the band on SDS-polyacrylamide gels is certainly a characteristic feature of the δ polypeptide (28, 30).

The alteration in the hydrodynamic properties of the pig brain [³H]gabapentin-binding protein, which occurs during purification, is almost certainly explained by the dissociation of the α_1 and β subunits. Takahashi *et al.* (28) found that the skeletal muscle $\alpha_2\delta$ subunit was associated with an $\alpha_1\beta\gamma$ heterotrimeric complex in solutions containing either 0.5% digitonin or 0.1% CHAPS. However, in 0.5% Triton X-100, the $\alpha_2\delta$ subunit was found to dissociate from the tripartite complex. Our hydrodynamic data suggest that the [³H]gabapentin-binding protein is associated with other proteins immediately following solubilization with 0.4% Tween 20, and that dissociation begins on the Q-Sepharose column. The presence of both free and complexed $\alpha_2\delta$ subunits may explain trailing of the [³H]gabapentin binding activity profile for this column.

Mechanism of Action of Gabapentin—All anticonvulsant drugs must ultimately exert their actions by modulating the activity of the basic mediators of neuronal excitability: voltage- and neurotransmitter-gated ion channels. Our data suggest that the $\alpha_2\delta$ Ca^{2+} channel subunit may be the critical target at which gabapentin exerts its antiepileptic action. This is supported by previous studies that have shown a correlation between the affinity of ligands at the [³H]gabapentin binding site and anticonvulsant activity (8). Other ligands acting at Ca^{2+} channels have been shown to possess anticonvulsant properties in animal seizure models. Flunarizine is effective against electroshock-induced seizures in rodents (31, 32); dihydropyridines prevent seizures elicited by pentylenetetrazol (31, 33), ethanol withdrawal (34), *N*-methyl-DL-aspartic acid, and the L-type Ca^{2+} channel agonist, BAY K 8644 (31). However, gabapentin is unique among Ca^{2+} channel ligands in that it acts at the $\alpha_2\delta$ subunit rather than at the α_1 subunit.

The physiological role of the $\alpha_2\delta$ subunit is not well understood at present. Co-expression of $\alpha_2\delta$ with the α_1 and β subunits is known to be required for efficient assembly and functional expression of Ca^{2+} channel complexes (21, 23, 26). Since the $\alpha_2\delta$ subunit appears to be common to all voltage-dependent Ca^{2+} channels (26, 35), it is conceivable that gabapentin modulates the activity of more than one type of neuronal Ca^{2+} channel. In mouse spinal cord neurons gabapentin blocked responses to BAY K 8644 (36), but in other studies gabapentin did not significantly affect L-, N-, or T-type voltage-dependent Ca^{2+} channels (37). However, given the structural diversity of Ca^{2+} channels, as revealed by molecular cloning studies (26, 35), data from a few electrophysiological studies should be interpreted with caution. At least six genes encoding Ca^{2+} channel α_1 subunits have been identified. Classes C, D, and S are sensitive to dihydropyridines (L-type), class B is sensitive to ω-conotoxin GVIA (N-type), and class A is sensitive to ω-agatoxin IVA (P-type). Class E is resistant to the agents listed above (B- and T-type channels). At least four genes encode β subunits, one gene encodes the $\alpha_2\delta$ subunit, and multiple splice variants of α_1, β and $\alpha_2\delta$ have been described (26, 35). The potential combinational heterogeneity of Ca^{2+} channels at the structural level is enormous. It is possible that gabapentin exerts *functional* effects only with particular combinations of subunits. Moreover, these effects may be observed only under conditions that mimic closely the excessive repetitive discharges that characterize clinical epilepsy. Further studies on the cellular electrophysiological actions of gabapentin, in a variety of systems, are required before the action of the drug at Ca^{2+} channels can be fully understood.

Finally, the data reported here show that the protein labeled by [³H]gabapentin in brain membranes is not the L-system transporter. However, the high affinity interaction of certain L-system substrates (*e.g.* L-leucine and L-methionine; Ref. 11) with the $\alpha_2\delta$ subunit is intriguing. To our knowledge the effects of neutral amino acids on the functional activity of voltage-dependent Ca^{2+} channels have not been investigated. However, endogenous ligands such as these presumably compete with gabapentin *in vivo* for the binding site on the $\alpha_2\delta$ subunit. This perhaps explains why the therapeutic concentration of gabapentin (2) is well above the K_D of the drug at the [³H]gabapentin binding site. We cannot say from present data whether the gabapentin binding site is located on the α_2 or the δ component, or whether it is extracellularly or intracellularly disposed. However, the heavy glycosylation of $\alpha_2\delta$ (24, 28) and weak labeling by hydrophobic photoaffinity probes (28) suggest that the bulk of the $\alpha_2\delta$ subunit is found at the extracellular surface. Further studies on the topology of the $\alpha_2\delta$ subunit and the precise location of the [³H]gabapentin binding site are required.

In summary, we have purified and characterized a high affinity [³H]gabapentin-binding protein from pig brain membranes. N-terminal sequencing has identified the protein as an $\alpha_2\delta$ subunit of a voltage-dependent Ca^{2+} channel. This conclusion is supported by tissue distribution studies, by hydrodynamic data, by heterologous expression of cloned $\alpha_2\delta$ cDNA in COS-7 and HEK cells, and by radioligand binding and immunoblotting studies on fractionated Ca^{2+} channel subunits. [³H]Gabapentin is the first ligand described that interacts with the $\alpha_2\delta$ subunit. We suggest that modulation of voltage-dependent neuronal Ca^{2+} channels may be important to the antiepileptic action of gabapentin.

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